

Photosynthetic Excitation Pressure Causes Violaxanthin De-epoxidation in Aging Cabbage (*Brassica Oleracea* L.) Leaves

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Abstract: The purpose of the present studies was analysis of the age induced changes in photochemical efficiency and xanthophylls cycle pigments of the primary cabbage (*Brassica oleracea* L. cv. Capitata f. alba) leaves. Photochemical efficiency of photosystem II (PS II) was studied by a pulse amplitude modulated chlorophyll fluorescence apparatus, chlorophyll concentration was analysis spectrophotometrically and xanthophyll cycle pigments were estimated by high-pressure liquid chromatography (HPLC). Leaf senescence was accompanied with a decrease both in chlorophylls concentration, the photochemical efficiency and rate constant for PS II photochemistry whereas non-photochemical parameters increased. Excitation pressure (1-qP) which is a measure of relative lumen acidification increased by 1.2× in aging leaves. The maximum quantum yield of PS II showed no significant change. The level of de-epoxidised xanthophylls increased but the concentration of mono- and di-epoxy xanthophylls decreased in aging leaves. A linear relationship between the excitation pressure and the de-epoxidation state of the xanthophyll cycle pigments and lutein, during the onset of senescence suggests that excitation pressure can be used as a sensor for monitoring the onset of senescence as well for the de-epoxidation state of the xanthophylls responsible for non-photochemical quenching in stressed leaves.

Key words: Cabbage (*Brassica oleracea* L.), violaxanthin cycle, excitation pressure, senescence, photosynthetic parameters.

Abbreviations: Ax, antheraxanthin; Car, carotenes; Chl *a* and *b*, chlorophyll *a* and chlorophyll *b*; DES, de-epoxidation state of violaxanthin; Fm, maximal fluorescence level in the dark; Fm', maximal fluorescence in the light; Fo, minimal fluorescence level in the dark; Fo', minimal fluorescence in the light; Fs, actual fluorescence level; Fv, variable fluorescence level in the dark; Fv/Fm, photochemical efficiency of PS II under dark adapted state; Fv'/Fm', quantum yield of PSII electron transport.

Q_A, primary quinone acceptor in PSII; L, lutein; Lx, Lutein-5, 6-epoxide; NPQ, non-photochemical quenching; PAR, photosynthetic active radiation; qN, rate constant for non-photochemical quenching; qP, rate constant for photochemical quenching; Vx, violaxanthin; VDE, violaxanthin de-epoxidase; Zx, zeaxanthin.

Leaf aging and senescence are used interchangeably in this study.

1. Introduction

Leaves develop from leaf primordia, undergo phases of several patterns formation during development to maturity, and after a productive photosynthetic period, leaf cells enter the phase of senescence otherwise

known as aging [1-3]. Leaf senescence is the most remarkable developmental event in plant life. There is an ordered metabolic shift from anabolism to catabolism, and sequential degradation of cellular structures [1-3]. Leaf senescence in higher plants is a type of apoptosis [1-3].

One of the important questions is how the senescence process is regulated in an orderly manner in leaves [1-3]. The useful and convenient senescence

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markers so far used widely are the changes in photosynthetic efficiency starting from primary photochemistry to carbon fixation [4]. Leaf yellowing or the loss of chlorophyll (Chl) is an external manifestation of leaf senescence [5-8]. This leads to an array of biochemical, biophysical and molecular changes in the chloroplasts resulting in the disassembly of the photosynthetic apparatus and concomitant decrease in the photosynthetic performance [4, 5]. The loss of PS II activity during leaf senescence is extensively studied leading to characterization of the site of damage in the electron transfer chain from water to the electron acceptors of PS II [4-8].

Senescing leaves efficiently adjust composition of their photosynthetic components to prevailing light environment [9]. They act as shade leaves, which have lower Rubisco content relative to their Chl level than the sun leaves [9]. The unshaded leaves showed a slower decrease in Chl and F_v/F_m than in the carboxylation activity [10]. These indicate that the stromal activity decreased faster than the thylakoid activity including light harvesting. Under conditions of lower activity of Calvin-Benson cycle, plants tend to absorb light beyond their competence for photosynthesis, and the electron transport components between PS I and PS II are reduced. This state of the thylakoid membrane is known as excitation pressure, which determines the redox state of plastoquinone pool [11]. The redox states of the electron transport components in thylakoid membranes affect several processes in plants including photosynthetic gene expression [12]. Chloroplasts developing under high excitation pressure are sun-type [13]. Therefore, it is likely that PSII excitation pressure during the leaf development affects photosynthetic activity of the leaf. However, it has not been investigated whether PS II excitation pressure also works as a sensor of changes in light environment during leaf aging/senescence.

When light is excessive, in all vascular plants and some species of algae, two de-epoxidised pigments are formed. One of them is completely epoxide free,

zeaxanthin (Zx) and another, monoepoxide antheraxanthin (Ax). These pigments are formed by de-epoxidation of violaxanthin (Vx) and they all are involved in a photoprotective process whereby excess absorbed excitation energy is dissipated thermally in the light-harvesting antennae of PSII. This process is called xanthophyll or violaxanthin cycle [14-17]. Vx is converted to Zx via Ax in a reaction catalyzed by the enzyme violaxanthin de-epoxidase (VDE) when the luminal pH decreases as a consequence of the light reactions, producing a higher proton gradient than can be utilized in CO_2 fixation [18, 19]. Such changes in the xanthophyll pool were also reported in some types of stress and stress tolerance mechanisms of plants [20, 21]. The retention of Zx plus Ax in photoinhibited leaves often correlates closely with sustained low PSII efficiencies measured as the F_v/F_m [22]. Such correlations have led to the suggestion that Zx plus Ax may be engaged for thermal energy dissipation under these conditions and may therefore be involved in the reduced PSII efficiencies observed.

Identification and characterization of the processes that regulate leaf senescence is crucial for the understanding of the underlying phenomena of leaf senescence and plant life cycle. Deciphering the mechanism of leaf senescence could facilitate the biotechnological applications to enhance plant productivity, post-harvest technology and stress adaptation [1, 3].

In the present study the authors followed the ontogenic changes of cabbage (*Brassica oleracea* L.) leaves, and the excitation pressure and the violaxanthin cycle pigments as sensors for leaf aging were investigated.

2. Experiment

Cabbage (*Brassica oleracea* L. cv. Capitata f. alba) seedlings were grown in hydroponic cultures, supplied with Hoagland nutrient solution under white light intensity of (PAR) $125 \mu\text{mol photons} \cdot (\text{m}^2 \cdot \text{s}^{-1})$, 10 h light period, 60-70% RH and temperature of $24 \pm 2^\circ\text{C}$

as described by Misra et al. [21]. Age induced changes in photochemical efficiency and pigments of the first (primary leaf) were monitored from 7 day to 16 day at 3 day intervals.

Photochemical efficiency of photosystem II (PS II) was studied by a pulse amplitude modulated chlorophyll fluorescence apparatus (PAM 101 with data acquisition software DQ, Walz, Germany) as described by Schreiber et al. [23]. The initial level of chlorophyll fluorescence (F_o) was measured with a dim red light modulated at 600 Hz. Saturating light intensity for F_m and F_m' was $4,500 \mu\text{mol photons (m}^{-2}\cdot\text{s}^{-1})$, and was 800 msec in duration, which was sufficient to achieve a stable maximum fluorescence yield. The maximal or optimal quantum yield of PS II photochemistry was calculated as $F_v/F_m = (F_m - F_o)/F_m$. The actual or effective quantum yield of PS II photochemistry (Φ_{II}) was measured in leaves illuminated with white light produced by a Schott light source (KL 1500) as $(F_m' - F_s)/F_m'$, where F_s is the steady state fluorescence level and F_m' is the maximal fluorescence level in the light. The non-photochemical quenching of chlorophyll fluorescence (NPQ) was calculated as $[(F_m/F_m') - 1]$ according to Bilger and Björkman [24]. To determine the rate constant of photoinactivation of PSII in the photochemical (q_P) and non-photochemical (q_N) quenching following calculation according to Genty et al. [25] was done: $q_P = (F_m' - F_s)/(F_m' - F_o) = \text{redox state of PS II with higher values indicating greater proportion of open or oxidised reaction center}$ and $q_N = (1 - F_v'/F_v) = q_N = (F_v - F_v')/F_v$ nonphotochemical quenching $\text{NPQ} = (F_m' - F_m)/(F_m)$ were also analysed.

Total chlorophyll content was estimated spectrophotometrically in 80% aqueous acetone extract as described by Arnon [26] (1949). Xanthophyll cycle pigments were estimated by high-pressure liquid chromatography (HPLC, Jasco, Japan) method as described by Latowski et al. [27]. The de-epoxidation state was calculated from the equation $[\% (Z_x + 0.5A_x) / (V_x + A_x + Z_x)]$. Xanthophyll pigments were

extracted from leaf samples, treated frozen in liquid nitrogen by solvent A containing: acetonitrile:methanol:water (36:4:1) using mortar and pestle, centrifuged for 10 min at $10,000 \times g$ in Microfuge Type 320. Supernatant was loaded to column (with 20 microliter loop volume, column: Nucleosil Gel 250 \times 4 m, 5 micrometer, TEKNOCROMA, Barcelona, Spain) and separated in solvent A with flow rate 0.7 mL/min, and after retention time of Z_x solvent was changed to methanol: ethyl acetate (340: 160) and rate of flow was 2 mL/min. The pigments detection was at 440 nm (Detecting light source: UV-970 Jasco, Tokyo, Japan). Peak area measurements were performed using a program Borwin Chromatography Software, Version 1.20, JMBS Developments, Le Fontanil, France. The maximum level of de-epoxidation state was measured for 1 h under saturating light ($2,000 \mu\text{mol}\cdot(\text{m}^{-2}\cdot\text{s}^{-1})$) condition [28-31].

3. Results

The cabbage leaves showed a gradual decrease in the Chl content from 7th day until 16th days of seedling growth (Fig. 1). Decrease in Chl content was already previously used as a base of the external manifestation of leaf aging [4, 6, 32]. Therefore the period from 7th day through 16 days of seedling growth was considered as leaf aging time and the changes in photosynthetic parameters and violaxanthin cycle pigments of these leaves were studied during that time.

The photosynthetic parameters under the dark adapted and light adapted state of aging cabbage leaves were analyzed by PAM Chl fluorescence measurements. During leaf aging the maximal fluorescence (F_m) decreased by 8% and minimal fluorescence (F_o) increased by 7% (Fig. 2).

This resulted in a decrease in the maximum quantum yield of PS II or the photochemical efficiency of PS II under dark adapted state, $F_v/F_m = [(F_m - F_o)/F_m]$ (Fig. 3A). The actual fluorescence quenching under light adapted state (q_P and Φ_{II}) also showed a sharp decrease in the

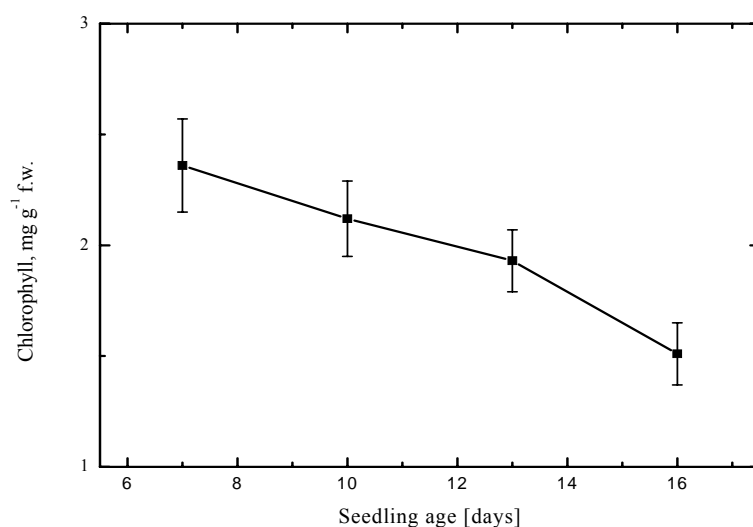


Fig. 1 Age induced changes in the total Chl content of cabbage (*Brassica oleracea* L.) leaves.

Each data point is an average (mean \pm SD, $n = 3$) of 3 separate experiments with 5 leaves each. The bars represent standard deviation of the mean. The same with Figs. 2 and 3.

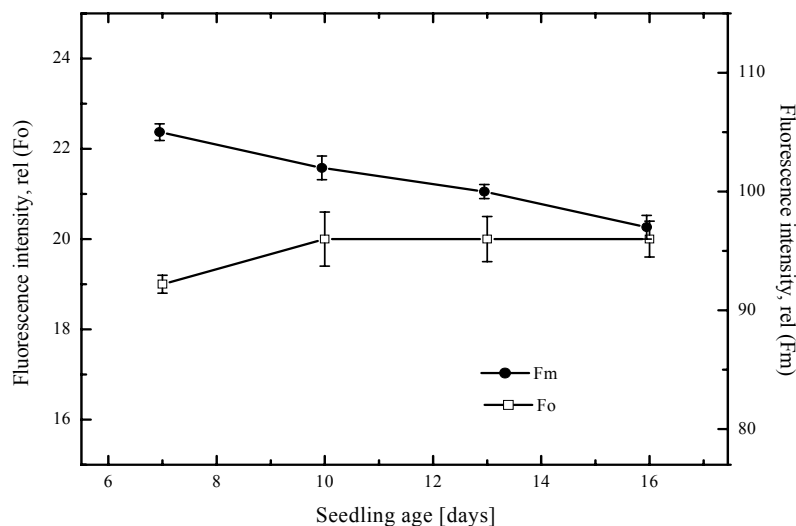


Fig. 2 Age induced changes in the fluorescence intensity Fo and Fm of cabbage (*Brassica oleracea* L.) leaves.

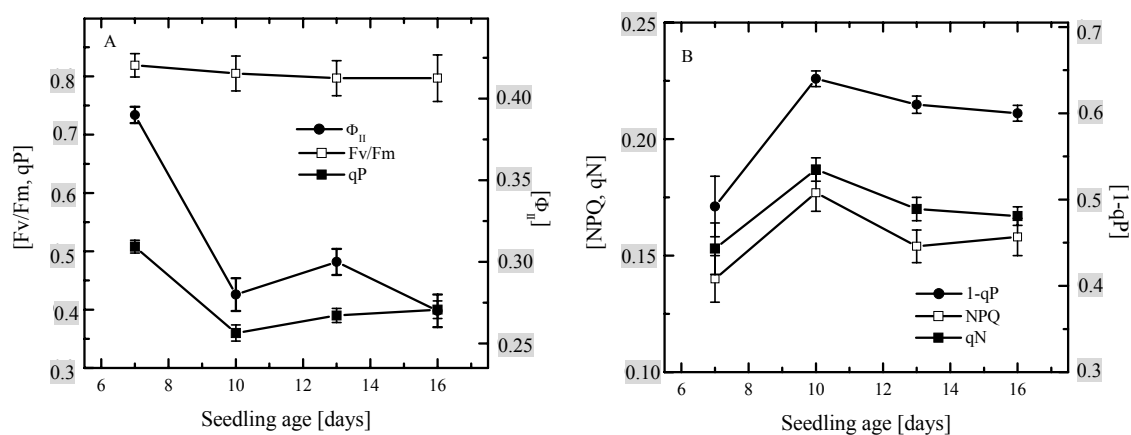


Fig. 3 Age induced changes in the photochemical (A) and non-photochemical (B) quenching parameters of cabbage (*Brassica oleracea* L.) leaves.

initial phase of leaf aging at 10 day (Fig. 3A). The photochemical efficiency of PS II (Φ II) in the light adapted state decreased by 31% and the rate constant for the photochemistry of PS II (qP) decreased by 21% (Fig. 3A). The parameter qP is a reflection of the redox state of Q_A in the light. A decrease in the qP indicates that Q_A becomes more reduced, and therefore the balance between reduction and re-oxidation of Q_A shifts towards reduction. This may either be due to a higher excitation rate of PS II or to a slower outflow of electrons towards the Calvin-Benson cycle. In contrast, the non-photochemical parameters of Chl fluorescence viz. rate constant for non-photochemical quenching (qN) and non-photochemical quenching (NPQ) as well as energy dissipation through non-photochemical means increased in aging leaves (Fig. 3B). On 16 day, the values for qN increased by 9% and that for NPQ increase by 13% of 7 day values (Fig. 3B). The excitation pressure (1-qP) which is a measure of the relative amount of the lumen acidification increased to 1.2x in aging leaves (Fig. 3B).

Analysis of the amount of the violaxanthin cycle pigments also showed a gradual and significant ($P < 0.5$) change during leaf aging (Fig. 4). The amount of Vx and Ax decreased but the amount of Zx increased about 2.5x in the aging leaves (Fig. 4A). The de-epoxidation state of xanthophyll cycle pigments

increased gradually and a 13 day onwards it reached 1.5x that of 7day old seedlings (Fig. 4B).

Lutein is most abundant comprising of 30-60% of total xanthophylls pigments and plays a structural role in LHC [18, 19, 33, 34]. Like violaxanthin cycle pigments, L undergoes epoxidation and de-epoxidation in the thylakoid membranes of cabbage primary leaves and it is also regulated by the redox state of the thylakoid [18, 19, 33, 34]. During leaf aging the level of L (the de-epoxide form) increased gradually and the amount of lutein-5,6-epoxide (Lx) decreased, leading to a gradual increase in the de-epoxidation state of L (Fig. 5).

The de-epoxidation of xanthophyll cycle pigments is regulated by the enzyme de-epoxidase and is well characterized [34, 35]. The epoxidation and de-epoxidation in plants is regulated by lumen acidification [19]. As the excitation pressure of photosynthetic PSII reactions are a measure of the redox state of quinone pool and the lumen acidification [11, 36], the relationship between the de-epoxidation state of violaxanthin cycle pigments [% ($Zx + 0.5Ax$)/($Vx + Ax + Zx$)] and de-epoxidised Lx [%L/(L+Lx)] with that of excitation pressure (1-qP) as depicted in Fig. 6 were analysed. The de-epoxidation of xanthophyll pigments showed a saturation kinetics with excitation pressure (Figs. 6A and 6C), which

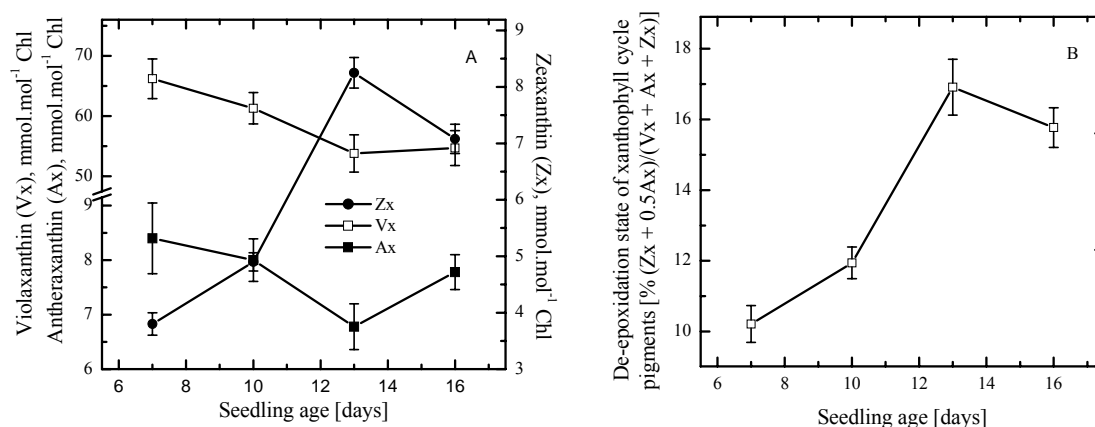


Fig. 4 Age induced changes in the xanthophylls cycle pigments (A) and their de-epoxidation state [% ($Zx + 0.5Ax$)/($Vx + Ax + Zx$)] (B) in cabbage (*Brassica oleracea* L.) leaves with the seedling age.

Each data point is an average of 2 separate experiments. The same with Fig. 5.

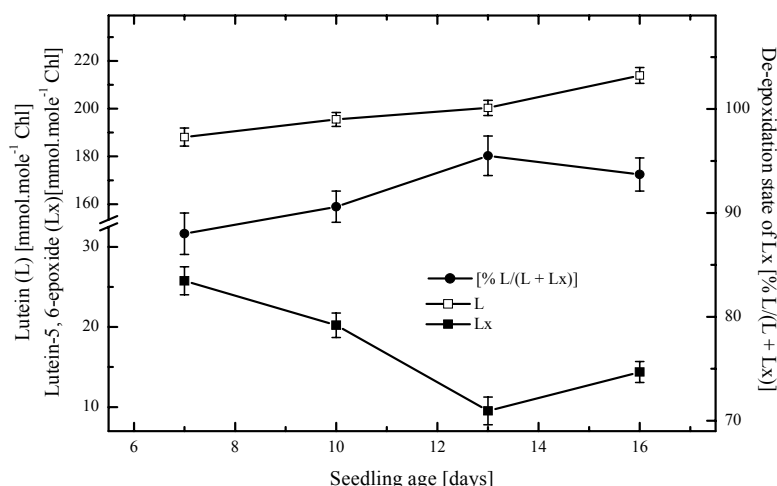


Fig. 5 Age induced changes in the lutein (L) and lutein-5, 6-epoxide (Lx) pigments of cabbage (*Brassica oleracea* L.) leaves. The de-epoxidation state of Lx as percent of total lutein [%L/(L+Lx)] (closed circles) is shown.

suggests that at lower de-epoxidation states, the excitation pressure could become linearly related or regulated proportionately by the regulation of quenching of excess dissipative energy by xanthophylls in aging leaves. However, after a critical concentration of the de-epoxidised xanthophylls can maintain the excitation pressure in aging leaves probably regulating the energy quenching to their maximum limit (Figs. 6A and 6C). The increase in the excitation pressure or the lumen acidification during aging of brassica leaves, de-epoxidation of xanthophyll pigments continued linearly (Figs. 6B and 6D).

This suggests that the de-epoxidase enzyme activity continues progressively during leaf aging in brassica and the substrates either violaxanthin or lutein epoxide cycle pigments do not become limiting during the leaf aging. There was a need to invoke thermal energy dissipation associated with xanthophyll pigments [13] in aging cabbage leaves due to an increase in the redox pressure.

4. Discussion

The changes in photosynthetic parameters and xanthophyll cycle pigments of aging cabbage leaves from 7 day through 16 days of seedling growth were studied. The initial fluorescence F_0 gradually increased and maximal fluorescence F_m gradually decreased

leading to a decrease in photochemical efficiency of PS II under dark adapted state, $F_v/F_m = [(F_m - F_0)/F_m]$ (Figs. 3 and 4). The increase in the F_0 is reported in the heat stressed leaves, in which increase of this parameter is attributed to a combination of processes such as (i) dissociation of light-harvesting complex II (LHC II) from the PSII complex and accumulation of inactive RCs of PS II [37, 38], (ii) reduction of Q_A in the dark [38], (iii) enhanced back electron transfer from Q_B to Q_A [39], and/or (iv) heat induced monomerization of LHC II trimers [40]. This might also be the reason for the increase in F_0 in aging cabbage leaves.

Moreover, a sharp decrease of q_P and Φ_{II} was observed in the initial phase of cabbage leaf aging at 10 day (Fig. 3). The parameter q_P is a reflection of the redox state of Q_A in the light [11, 25] and the decrease of the value of this parameter indicates that Q_A becomes more reduced, and therefore the balance between reduction and re-oxidation of Q_A shifts towards reduction. This may either be due to a higher excitation rate of PS II or to a slower outflow of electrons towards the Calvin-Benson cycle [11]. The non-photochemical quenching of Chl fluorescence, q_N and NPQ, and the excitation pressure measured as $(1-q_P)$ increased significantly in aging leaves (Fig. 3). The aging induced inhibition of PS II photochemical

efficiency with a subsequent decrease in the quantum efficiency of light energy utilisation by PS II and charge separation of primary charge pairs in PS II [4, 6, 7] could lead to the absorption of light beyond their competence for photosynthesis. Excess light absorbed by the light-harvesting chlorophyll-protein complexes, LHCs of PS II, is dissipated as heat through various non-photo chemical processes commonly known as qN or NPQ [11, 35]. A decrease in the photochemical utilisation of absorbed quanta and increase in the dissipation of excess light as heat is reported in the aging leaves [4, 5]. The redox states of the electron transport components and the excitation pressure thylakoid membranes affect several processes in plants including photosynthetic gene expression [12]. Huner et al. [13] reported that chloroplasts developing under high excitation pressure are sun-type. However, it has

not been investigated whether PS II excitation pressure also works as a sensor of changes for structure/function relationship during leaf aging/senescence.

The de-epoxidised xanthophyll cycle pigments Zx and Ax are formed from Vx (epoxide form) when light is excessive, and they are involved in a photoprotective process whereby excess absorbed excitation energy is dissipated thermally in the light-harvesting antennae of PSII [14-16]. The retention of de-epoxidised Zx plus Ax in photoinhibited leaves often correlates closely with sustained low PSII efficiencies measured as the F_v/F_m [22]. Such correlations have led to the suggestion that Zx plus Ax may be engaged for thermal energy dissipation under these conditions and may therefore be involved in the reduced PSII efficiencies observed. Presumably, photo-transformation of Vx and binding of protons to the LHCs act synergistically to

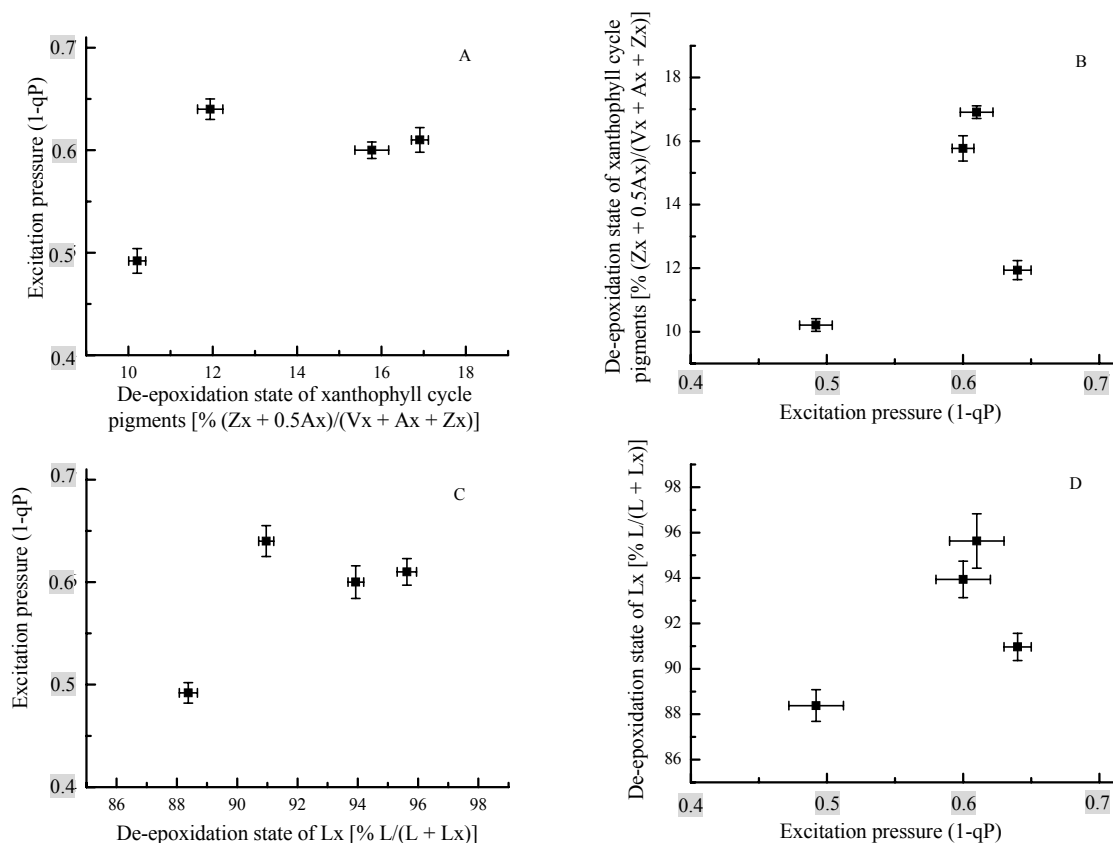


Fig. 6 Analysis of the relationship between de-epoxidation state of xanthophyll cycle pigments (A, B) and de-epoxidation state of lutein-5,6-epoxide (Lx) (C, D) with photosynthetic excitation pressure. The saturation kinetics (A, C) and linear relationship between the excitation pressure and de-epoxidation states (B, D) at the onset of senescence is a clear-cut indication of the sensory mechanism in lumen acidification and xanthophyll pigments de-epoxidation in brassica leaves.

induce a conformational change, that is necessary for thermal energy dissipation. However, *npq* mutants defective in heat dissipation processes in *Arabidopsis* and *Chlamydomonas* suggest that during long-term adaptation of plants to high light, other protective processes can compensate for these defects [28]. In such a case plants adapted to stressful environment, where they dissipate heat under stress, might play both NPQ dependent and NPQ independent processes. Also the xanthophyll cycle pigment dynamics may be altered as per the NPQ related processes.

The increase in the level of de-epoxidised xanthophylls (Zx and L) with an increase in NPQ in aging leaves might signify protective function of these xanthophylls during leaf aging (Figs. 3-6). This corroborates with the findings described for the photoinhibited leaves [33] and leaves from stress sensitive plants [41]. The de-epoxidation of Vx to Zx is mediated by the reductant ascorbate [35, 42] proposed that the size of the Vx fraction available for de-epoxidation is related to the redox state of the PQ pool. The more the reduced PQ pool the greater Vx availability for de-epoxidation. A gradual and significant decrease in Vx in the aging cabbage (*Brassica oleracea* L.) leaves (Fig. 4A) clearly demonstrates the fact that it is sensing the increased reduction of the PQ pool *in vivo* through the increase in the excitation pressure (1-qP) (Fig. 3B). The steady state level of Vx and Zx do not only depends on the availability of Vx for de-epoxidation, but also on the ratio of epoxidation to de-epoxidation at each light intensity. The reverse reactions are catalyzed by an epoxidase [42]. The back reaction rate is decreased with the increased light intensity [42]. A faster decrease in the PS I activity [32] and RUBISCO activity [4] compared to that of PSII activity in aging leaves could also lead to a decrease in the production of NADPH and the increased excitation pressure (Fig. 3B) can facilitate lumen acidification in aging brassica leaves. This could lead to the increase in the de-epoxidation of xanthophylls pigments in aging

leaves. Increase in the xanthophyll cycle pigments was also reported under natural senescence of *Pistacia* leaves [38], white clover [39] and in wheat leaves [43, 44] in the late stage of senescence. But in this study it is shown that de-epoxidation of xanthophyll pigments (Figs. 4 and 6) starts with the onset of leaf aging or senescence as measured by a decrease in Chl content (Fig. 1).

5. Conclusion

It is concluded from this study that (i) PS II excitation pressure also works as a sensor of changes in light environment during leaf aging/senescence; (ii) there is a significant change in the xanthophyll cycle pigments in aging/senescent leaves growing under low light conditions, also. So excitation pressure and/or the xanthophyll cycle pigments can act as sensors for cabbage leaf aging. The linear relationship between the excitation pressure and the de-epoxidation state of the xanthophyll cycle pigments, during the onset of senescence states that excitation pressure can be used as a sensor for monitoring the onset of senescence as well for the de-epoxidation state of the xanthophylls responsible for non-photochemical quenching in stressed leaves.

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